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13. ABSTRACT *(Maximum 200 words)*

The performance and behavior of enzymes in non-aqueous environments has been explored under a variety of conditions, including high temperature and pressure. This work is both of fundamental interest, for providing information on enzyme behavior in non-aqueous environments, as well as of industrial importance, in that commercial processes which could benefit from the use of enzymes often operate in organic solvent, or at temperatures and pressures far from ambient.

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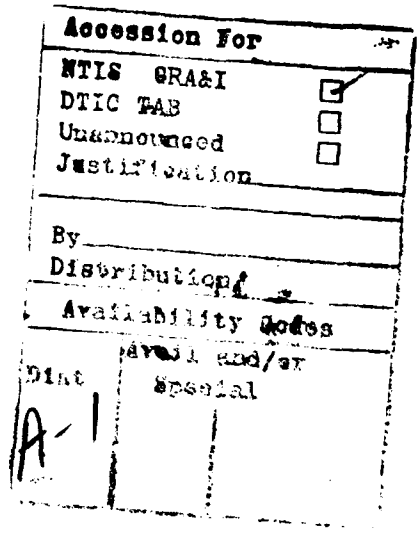
The work was directed at several topics within this general theme, including (a) biocatalysis in supercritical fluids; a study of the effect of smooth variations in solvent properties on enzyme activity, (b) biocatalysis in microemulsion systems, a study of the effect of microemulsion system variables on enzyme solubilization and activity, (c) Spectroscopic investigation of subtilisin in organic solvent; a study of the conformation of an enzyme in organic solvent as compared to its conformation in aqueous solution.



BIOCATALYTIC PROCESSING OF POLYMERS IN SUPERCRITICAL FLUIDS

ARO FINAL REPORT: DAAL03-90-G-0145

FORWARD:

As a forward we have chosen to include the original abstract from our proposal:

The use of enzymes for synthesis and degradation of polymers in supercritical fluids is proposed. Specifically, the identification of enzymes for the synthesis of acrylates (the important monomers which form the poly(acrylates)), synthesis and depolymerization of the polyesters poly(ethylene terephthalate) and poly(ethylene glutarate), and peptide synthesis are proposed. Proteases and lipases will be used to catalyze transesterification of acrylates in supercritical carbon dioxide. This model system will be investigated in detail in an attempt to relate the structure and function of an enzyme to the temperature, pressure, and solvent in which it is placed. **The aim will be to establish a set of general guidelines for the optimization of enzymatic processes in supercritical environments.** Using these guidelines the proposed research will establish the feasibility of biocatalytic synthesis and depolymerization of aromatic and aliphatic polyesters. In addition, the use of lipases and proteases for peptide synthesis will also be evaluated. In each case, the effect of solvent, temperature and pressure, on enzyme specificity, activity, and stability, will be determined. While this research is exploratory in nature, research in the last five years has laid the ground rules for the development of processes such as those that we propose.

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STATEMENT OF PROBLEM

The performance and behavior of enzymes in non-aqueous environments has been explored under a variety of conditions, including high temperature and pressure. This work is both of fundamental interest, for providing information on enzyme behavior in non-aqueous environments, as well as of industrial importance, in that commercial processes which could benefit from the use of enzymes often operate in organic solvent, or at temperatures and pressures far from ambient. Our work is directed at several topics within this general theme, including (a) biocatalysis in supercritical fluids; a study of the effect of smooth variations in solvent properties on enzyme activity, (b) biocatalysis in microemulsion systems; a study of the effect of microemulsion system variables on enzyme solubilization and activity, (c) Spectroscopic investigation of subtilisin in organic solvent; a study of the conformation of an enzyme in organic solvent as compared to its conformation in aqueous solution. We shall present briefly some details from work in these areas, and more detailed information is provided in the attached manuscripts.

RESULTS

We have shown that biocatalytic (a lipase from *Candida cylindracea*) transesterification can be conducted in a variety of organic solvents, at a rate which varies as the properties of the solvent vary. For example, the reaction of 2-ethyl hexanol with methyl methacrylate proceeds faster in more hydrophobic solvents, although the relatively sluggish activity observed in hydrophilic solvents can be raised via the addition of water. Further, we have conducted a wide-ranging study of the effect of supercritical fluid properties

(both molecular and density-based) on the activity of lipase (from *Candida cylindracea*) in the transesterification of methyl methacrylate by 2-ethyl hexanol. Significant results include:

* Although carbon dioxide is without question the most widely-used supercritical fluid owing to its environmentally-benign characteristics, we have shown that CO_2 inhibits the lipase (from *Candida cylindracea*). This inhibition is completely reversible upon removal of the enzyme from CO_2 exposure, and is likely to bethe result of formation of reversible covalent complexes between CO_2 and free amine groups on the surface of the enzyme.

* In general, the enzyme follows Michaelis-Menten kinetics in supercritical ethane, (and likely also in fluoroform, sulfur hexafluoride, and ethylene) and activity can be controlled through changes in pressure. Using a computer database for supercritical fluid properties (density, viscosity, dielectric constant, solubility parameter) developed in our laboratory, we have shown that the activity of the enzyme correlates well to the dielectric constant of the fluid, which itself is a strong function of pressure for supercritical fluids. Through examination of reaction rates in solvents such as sulfur hexafluoride and fluoroform, we have been able to examine the effect of smooth changes in dielectric constant over a range of 1 to nearly 10.

* Using a set of standard equations we have calculated the role of internal and external mass transfer in limiting the rate of enzyme-catalyzed reactions in anhydrous organic solvents and supercritical fluids. We have shown that enzyme particles suspended in anhydrous organic solvents will be subject to increasing diffusional limitation as the enzyme activity and particle size increase. Using particle dimensions measured by scanning electron microscopy, we have prepared a series of graphs which will enable investigators to determine whether their combination of particle size and

activity will result in internal or external diffusional limitations. We have shown that supercritical fluids can be expected to enhance the activity of enzymes in non-aqueous environments as a result of the high diffusivity of the bulk solvent. The plots also clearly indicate that enzyme particles in supercritical fluids require nearly two orders of magnitude less agitation than those suspended in conventional solvents in order to overcome any external mass transfer limitations.

We have also described, for the first time, the ability of a polyoxyethylene sorbitan trioleate-isopropanol microemulsion in hexane to solubilize pure proteins. The dependencies of cytochrome C extraction and buffer solubilization by the reverse micellar system on ionic strength of the aqueous phase, detergent concentration, and co-surfactant concentration have been reported. Increases in ionic strength result in decreased recovery of protein in the organic phase, whereas increases in detergent concentration and co-surfactant concentration result in increased extraction. In addition, subtilisin (a serine protease) has been shown to be active in this microemulsion. Further the activity of the enzyme can be regulated by the water content of the micelles, enabling control of enzyme activity by "solvent engineering". This study has now been extended to demonstrate the first solubilization of an enzyme in a near-critical propane system containing Tween-85. Once again, cytochrome C and subtilisin retain their functional properties in the system, and more interestingly the degree of protein solubilization is dependent on pressure.

We have also initiated a study on the activity of phosphotriesterase in reversed micelles. The micelle system under study in our group uses the non-

ionic surfactant, polyoxyethylene sorbitan trioleate (Tween 85), which forms stable microemulsions in hexane with isopropanol or ethylene glycol as a cosurfactant. Tween 85 is non-toxic, biodegradable and has been used as an additive in fertilizers[56]. It was found that the stability of the enzyme in the micelles was enhanced over that in buffer. Presently, studies with phosphotriesterase involve a characterization of the micelle system for the degradation of organophosphorus pesticides and nerve gases. The specific activity of the enzyme approaches the value in buffer as the micelle size is increased, while the apparent saturation constant (K_m) of the enzyme is increased in the micelles. The stability of phosphotriesterase in the micelles is comparable to that in buffer. Characterization studies on the micelle system have included dynamic light scattering (DLS) for determination of apparent micelle size, and the preparation of phase diagrams. Finally, nuclear magnetic resonance spectroscopy (NMR) is being used to determine the partition coefficients of the cosurfactant in the micelle system. The chemical shift of isopropanol is significantly different in water and in hexane. The observed shift in the micelles is dependent on the relative amounts of alcohol in the hexane, water and surfactant volumes. Kinetic data for buffer and in micelles at different water contents are presented below.

	PARAOXON			
W_o	V_{max} mmol/mg/min	K_m (mM)	k_{cat} (s-1)	k_{cat}/K_m
6.9	107 ± 12	14.3 ± 3.4	70.3 ± 7.9	4.9
30	430 ± 28	28.9 ± 3.8	283 ± 18.4	9.8
45	556 ± 80	31.2 ± 8.2	366 ± 53	11.8
69	992 ± 149	34.8 ± 9.0	653 ± 98	18.8
86	1,198 ± 131	33.3 ± 6.7	789 ± 86	23.7
125	1,753 ± 259	29.5 ± 8.3	1150 ± 170	39.1
Tris-HCl	1,686 ± 42	0.019 ± 0.001	1,110 ± 28	58,421
	PARATHION			
5.3	2.88 ± 0.36	33.8 ± 7.3	1.9 ± 0.24	0.056
7	4.2 ± 0.29	51.1 ± 5.2	2.77 ± 0.019	0.054

81	23.7 ± 5.2	91 ± 29	15.6 ± 3.4	0.17
Tris-HCl*	2850 ± 540	0.35 ± 0.08	1870 ± 360	5,340

A complete understanding of structure-environment relationships requires the application of all possible techniques which provide information about protein structure. EPR and NMR are excellent techniques for investigating local regions of the protein. FT-IR, however, is a widely accepted method for the study of global protein structure in a variety of environments. The use of infrared spectroscopy in the study of proteins was pioneered by Elliott and Ambrose (for detailes see attached manuscript) and has been extensively applied since. The IR spectra of polymers such as proteins can be interpreted in terms of vibrations of structural repeat units. The vibration of a single repeat unit such as an α-helix or β-sheet can be separated from an otherwise complicated spectrum, enabling quantification of secondary structure. Nine groups of vibrational frequencies, manifested as characteristic bands in FT-IR protein spectra, have been identified. Of these, Amide I and Amide II are the most useful probes of protein structure. FT-IR is also readily applicable to the study of enzymes in anhydrous environments since there is no difference in results for solubilized and insoluble proteins. In fact, a major drawback in the use of FT-IR for protein structure analysis is the very strong absorption of water in the spectral region of interest, and thus non-aqueous FT-IR of proteins allows significantly more accurate analysis of typical amide vibrations. Based on a detailed study of conformationally sensitive infrared absorption frequencies we can detect any changes in protein secondary structure arising from altered environments of the protein. From work partially funded by ARO, we have concluded that there is no change in the secondary structure of subtilisin and myoglobin upon exposure

of powdered preparations of the enzyme to carbon tetrachloride and mineral oil. In addition, the data for azidometmyoglobin suggest that there is no significant change in the protein structure around the heme binding site, although there may be some solvent interactions affecting the band widths of the azide antisymmetric stretch.

The uses of pure enzymatic and chemoenzymatic strategies for a variety of polymer syntheses have received much attention recently. Optically active polyesters, polyacrylates, polyamides, conductive polyaromatics and sugar based polymers can be synthesized using enzymes suspended in conventional organic solvents. The molecular weight and molecular weight distribution of these polymers are, however, often difficult to predictably control. Since the solubility parameter of supercritical fluids is pressure dependent, it follows that the solubility of a given molecular weight polymer chain can be altered by manipulating the pressure of a supercritical fluid. Indeed, as molecular weight increases, solubility will decrease at constant pressure. Therefore, if the catalyst for a polymer synthesis is insoluble, the chain growth of the polymer can be terminated by adjusting the pressure in such a manner that the growing chain is no longer soluble (an insoluble catalyst cannot react with an insoluble substrate). The precipitated polymer would also have a low dispersity, and its molecular weight can be controlled by changing the pressure. As the pressure increases, so should the molecular weight of the polymer.

The principle for the strategy we propose resides in the ability of supercritical fluids to separate materials on the basis of their molecular weight. Using a supercritical fluid extractor kindly donated by Hewlett Packard, we have extracted a polymer sample of high dispersity at different

pressures of supercritical carbon dioxide. The extraction of poly(1,4-butylene adipate) with an average molecular weight of 10,000 and dispersity of 3.66 was performed. As the extraction pressure increases, the molecular weight of the extracted polymer also increases, indicating that the solubility of the polymer is proportional to pressure. Table 1 demonstrates that the enzyme-catalyzed synthesis of the same polymer can also be controlled by pressure-induced changes in solvent physical properties in fluoroform. While the dispersity remains low during an increase in pressure, the average molecular weight of both the soluble and precipitated polymer increases gradually. We are currently investigating the ability of enzymes to synthesize larger polymers in supercritical fluids.

Table 1: Effect of pressure on molecular weight and dispersity during lipase-catalyzed polymerization in supercritical fluoroform.

Pressure (psi)	Maximum Molecular Weight of the Soluble Polymer	Average Molecular Weight and Dispersity of the Soluble Polymer	Average Molecular Weight and Dispersity of the Precipitated Polymer
900	[a]987	[a]937 (1.07)	[a]1020 (1.02)
1600	1424	1037 (1.11)	1677 (1.03)
2400	2586	1371 (1.18)	2774 (1.03)
3000	2849	1762 (1.23)	3357 (1.05)

Table 1 legend: The alcoholysis of bis(2,2,2-trichloroethyl) adipate by 1,4-butanediol catalyzed by porcine pancreatic lipase suspended in fluoroform at 50 °C. The error in determination of polymer molecular weights is ±5 %.

[a]The extractions for the biocatalysis experiment performed at 900 psi were performed in a supercritical fluid extractor in which the minimum pressure achievable is 1200 psi.

Highlights from ARO Supported Research

1. Demonstration of control of polymer molecular weight during synthesis in supercritical fluids.
2. Imaging of enzyme powder hydration with electron microscopy.
3. Demonstration of biocatalysis in an inorganic non-aqueous solvent (sulfur hexafluoride).
4. Demonstration of structural integrity of proteins in anhydrous solvents using FT-IR.
5. Solubilization of proteins in non-ionic microemulsions in near-critical propane.
6. Demonstration of enzyme activity of soluble proteins in compressible fluids.

PUBLICATIONS AND ABSTRACTS ARISING FROM ARO SUPPORT

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